PROJECT 2

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PROJECT 2

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.
110 EAST 50TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: 6/30/75

1. Principal Investigator (give title and degrees):
Margit Hamosh, Ph.D. Assistant Professor of Anatomy

2. Institution & address:
Georgetown University
37th & O Sts., N.W.
Washington, D.C. 20007

3. Department(s) where research will be done or collaboration provided:
Anatomy, Physiology and Microbiology

4. Short title of study:
SMOKING AND LUNG DEVELOPMENT: A PROGRAM PROJECT
PROJECT # 2, SMOKING AND THE BIOCHEMICAL DEVELOPMENT OF THE LUNG

5. Proposed starting date: 1/1/76

6. Estimated time to complete: 5 years

7. Brief description of specific research aims:
SMOKING AND THE BIOCHEMICAL DEVELOPMENT OF THE LUNG. This project has two main thrusts: 1) to establish the maturation of biochemical pathways in the prenatal and postnatal period and 2) study selected enzymes the same way. The effect of exposure to cigarette smoke on the biochemistry of developing lung will be a major component of this study. Rodent and primate lungs will be studied at various phases of their development as to their ability to incorporate radio-labeled substrates for protein, carbohydrate and lipid synthesis. The biochemical findings will be correlated to functional studies with particular emphasis on lung mechanics in isolated lungs. The biochemical consequences of smoke exposure and structural alteration will also be studied.

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PROJECT 2 Page 1

Smoking and the biochemical development of the lung

Background The association between smoking and lung disease was always based on the assumption that the irritant effect of the smoke produces mechanical or chemical changes in the bronchial epithelium which ultimately leads to either narrowing or disruption (chronic bronchitis and emphysema) on the one hand, or metaplastic, oncogenic changes which ultimately result in bronchogenic carcinoma. Most of this association is purely statistical and the mechanism of cause-effect relationship has not yet been established satisfactorily. Since all smokers are equally exposed to the irritating effect of smoke, it is likely that host factors or some adjuvants play an equally important part in the pathogenesis of the disease entities associated with smoking. Indeed, a number of recent observations (1,2,a,b) indicate that hereditary ($alpha_1$ anti-trypsin deficiency), acquired (childhood bronchiolitis) and environmental (air pollution) factors are major determinants in the severity of lung disease developing in association with smoking. One would therefore speculate that the development of chronic lung disease is multifaceted with smoking representing one facet which might become deadly when associated with the other facets as well.

Virtually nothing is known about the influence of cigarette smoke on the developing lung. As a matter of fact, very little is known about the influence of almost anything on the developing lung. Recent work by Lynn Reid (3), Green and Mead (4) and others (5), suggests the intriguing possibility that developmental or maturation arrest, or nonisotropic development of the lung might be responsible for predisposing the lung to the noxious effects of environmental irritants; however, it is equally tempting to specualte that: a) the environmental factors are responsible for the developmental defects, or b) the developmental defects are due to inherent or acquired biochemical disturbances. Therefore, the central theme of this project is the determination of the basic biochemical processes during the development of the respiratory system and the effect of cigarette smoke on the course of this development. Recent work has shown that smoking by the mother exerts a retarding influence on fetal growth (6, a) and leads to a greater number of unsuccessful pregnancies due to either still birth or neonatal death (7), and that "passive" smoking by the child might increase the incidence of respiratory infections (8). If these observations are correct there are two possible ways in which smoking could affect the host: a) it could change the "substrate" for environmental invasion, b) it could weaken the defenses, such as the immune apparatus, or mucociliary clearance. We would like to explore the first possibility and see whether exposure to smoke of developing animals will change their biochemical processes and maturation. The embryonal and cellular responses are the subject of Project 3.

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Specific Background It is increasingly clear that the lung, in addition to its primary role in gas exchange, is a metabolically active organ. Studies during the past few years have shown that the lung consumes oxygen (9,10), utilizes energy yielding metabolites (11,12), and is active in the synthesis of metabolites needed to maintain its structural and functional integrity (12,13). There is a general lack of standardization of the methodology employed in the study of lung metabolism, (see review ref. 13) and as a result we have only a sketchy picture of the biochemical ability of the lung. The only lung component that has been studied thoroughly is the surfactant complex (14,15,16). Indeed, most of the biochemical research in recent years has concentrated on the study of the composition structure and biosynthesis of surfactant (see reviews, ref. 14,15,16,17).

If our knowledge of lung biochemistry is spotty at best in the adult of a number of species, it is almost nil when we approach the perinatal and early developmental period. Here again, the overwhelming importance of the surfactant system in early post natal life, led to in depth investigation of the synthetic pathways of dipalmitoyllecithin (the chief component of surfactant) (16,18,19). There is at present a considerable discrepancy between our knowledge of the anatomical development of the lung and that of its biochemical development; the first has advanced considerably during the last few years, as a result of the elegant studies of Reid (20), Boyden (21), Weibel (22), and their collaborators.

Lung maturation is generally determined by the ability of the pre and immediately post natal lung to synthesize and maintain an adequate concentration of alveolar surfactant (19,23,25). It has been established that the fetal lung reaches this stage a few days to a few weeks before birth, depending on the length of the gestation periods of the species (16,19,23-25).

Lung metabolism (with the exception of surfactant (16,19) and collagen synthesis (26) has been studied chiefly in adult animals. Glucose metabolism in the lung proceeds via glycolysis, Krebs cycle and pentose phosphate pathway (10,26); the lung produces relatively large amounts of lactate even when the PO_2 in the tissue is high (10,27). The pentose phosphate pathway which is a source of NADPH is especially important in protecting the lung from oxygen toxicity (28). Energy generating and utilizing pathways in lung tissue have only recently come under investigation (29). We have very limited information on protein synthesis in the adult rat lung (13). We are only starting to obtain information about the detoxifying ability of the lung. The lung has a special role in this respect since it is exposed both through the airways and through the vascular bed to a large number of potentially toxic substances. The role of mucociliary clearance (30) and of alveolar macrophages (31) in the protection of the lung are under active investigation. The role of the capillary endothelial cell has been largely overlooked and is emerging only recently as an active metabolic unit with special enzymatic functions in the uptake of circulating metabolites (32) and in the handling of bioactive substances (33,34).

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This research project sets several aims:

1. To evaluate the major metabolic pathways in the lung during pre-and post-natal development in the experimental animal (rats, rabbits, guinea pigs, mice, primates).

2. To study individual enzymes of special importance to intermediary metabolism such as:

   a. Lipoprotein Lipase, the enzyme that catalyzes and regulates the uptake of circulating triglyceride-fatty acids by the lung (32)
   b. Glucose-6-phosphate dehydrogenase, a key enzyme of the pentose phosphate pathway which maintains a steady supply of NADPH.

3. To study the effect of exposure to digarette smoke prenatally (i.e.: exposing the mother) or postnatally on the biochemical activity of the developing lung. In addition to the evaluation of the major metabolic pathways described in sections 1 and 2 we will measure enzyme activities or tissue components that could affect resistance or susceptibility to the ingredients of cigarette smoke.

4. To study the metabolic pathways of human lung - adult and newborn and correlate the findings to the smoking history of the subjects (adults) or the mothers (newborns).

EXPERIMENTAL DESIGN: The following parameters will be studied using as experimental preparation fresh lung slices: (We have chosen the tissue slice method since it is the least time consuming and according to recent studies (13,27) has an identical glucose metabolism as that of the isolated perfused lung.)

MAJOR METABOLIC PATHWAYS

1. Glucose metabolism. Uptake and utilization of glucose will be studied by measuring the amount of ^{14}C labelled glucose metabolized to $^{14}CO_2$ and lactate, and incorporated into tissue lipids.

The relative amount of glucose metabolized through glycolysis and Krebs cycle or through the pentose phosphate pathway will be measured by the use of 1 ^{14}C and 6 ^{14}C glucose substrate. The ratio of lactate to pyruvate production will be taken as a measure of the cytoplasmic redox state of the tissue; in addition the concentration of ATP and especially the ATP/ADP ratio will be measured.

2. Protein synthesis will be measured by the incorporation of labeled amino acids (leucine) into lung protein.

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3. Glucoprotein synthesis will be measured by the incorporation of labeled glucosamine into lung and tracheal glucoproteins.

4. Lipid metabolism Biosynthesis of lipids (neutral lipids and phospholipids) will be measured by the uptake and incorporation of labeled fatty acids and choline into tissue lipids.

If any changes will be found in the above described pathways during lung development, we will study the individual enzymes with the aim of localizing the specific change.

2. INDIVIDUAL ENZYMES OF SPECIAL IMPORTANCE TO INTERMEDIARY METABOLISM

a. Lipoprotein Lipase

During the transition between intrauterine to post natal life, there is in general a switch from the use of glucose as the main energy source to the use of other substrates, chiefly fatty acids. The ability of a tissue to utilize circulating fatty acids (which are present in the blood as triglyceride-fatty acids in chylomicrons and very low density lipoproteins) is directly related to the activity of lipoprotein lipase, an endothelial enzyme that catalyzes and regulates the uptake of blood triglyceride-fatty acids. We have recently described the enzyme in adult lung (32). The ability of the neonatal lung to utilize circulating triglyceride-fatty acids will be measured as a function of lipoprotein lipase activity.

b. Glucose-6-phosphate Dehydrogenase (G-6-P D), a key enzyme of the pentose phosphate pathway will be measured; this pathway is especially important in maintaining a steady supply of NADPH. An increase in G-6-P D activity has recently been linked to the development of tolerance to oxygen exposure in rats (28)

3. THE EFFECT OF EXPOSURE TO CIGARETTE SMOKE PRE-AND POSTNATALLY ON THE BIOCHEMICAL ACTIVITY OF THE DEVELOPING LUNG

a. Aryl hydrocarbon hydroxylase (AHH) a microsomal mixed function oxidase that metabolizes benz(a) pyrene and other polynuclear aromatic hydrocarbons present in tobacco smoke (35-37). It is of special interest to follow possible changes in the enzyme activity in lungs of fetuses whose mothers were exposed to cigarette smoke.

b. The content of cytochrome P-450 in lung.

c. The effect of antioxidants, such as Vitamin C on the prevention of changes caused by exposure to cigarette smoke.

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4. EFFECT OF CIGARETTE SMOKE ON THE METABOLIC PATHWAYS OF HUMAN LUNG (ADULT AND NEWBORN)

The Experimental Design will be similar to that of Sections 1,2 and 3.

Human Lung Specimens of adult human lung will be obtained during surgery. During the 5 year period of this research project, we hope to accumulate a large enough sample of lungs from smokers and non-smokers.

Newborn lung will be obtained at autopsy. We have the full cooperation of the Department of Neonatology, Georgetown University Hospital, and are confident that the specimens will be obtained immediately after death. Details of the smoking habits of the mothers will be obtained.

MATERIALS AND METHODS

Animals Rats, mice, guinea pigs and rabbits will be sacrificed at several time intervals from the beginning of the last trimester of pregnancy until parturition. The uteri will be removed and placed in cold Krebs Ringer bicarbonate solution; the fetuses will be decapitated and the lungs will be removed and stored in cold buffer until all the specimens have been collected.

Fresh fetal primate lung will be a gift from the NIH primate colony through the courtesy of Dr. Phillip M. Farrell, Chief, Section on Developmental Biology and Clinical Nutrition, Neonatal and Pediatric Medicine Branch, NICHD.

Analysis of tissue components The possible changes in lung composition during maturation and after exposure to cigarette smoke in experimental animals and man will be studied by measuring the concentrations of the following components: protein, DNA, RNA, lipid (total lipid, neutral glycerides and phospholipids).

Exposure to cigarette smoke

1. Pregnant animals

a. Pregnant animals will be exposed to smoke in the Walton Horizontal Exposure smoking machine developed under CTR contract by Process and Instruments Corporation, Brooklyn, New York. The animals will be divided into 4 groups. One group will be exposed to cigarette smoke (1 or 2 Kentucky reference cigarettes IAI or IRI daily) for the whole length of pregnancy; the second group will be exposed during the first trimester only; the third group during the last trimester of pregnancy only; the fourth group will be "chronic" smokers exposed for variable periods of time before pregnancy

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and throughout pregnancy. The animals will be allowed to deliver spontaneously; the composition and metabolic activity of the lung will be studied in the offspring of the four groups. The newborn animals will be divided into several groups and studied from birth until weaning (age 3 weeks). In addition to lung metabolism we will record size, weight and weight gain in the offspring of smoking mothers and compare it to non-smoking controls. For some experiments the pregnant rats will be sacrificed during the last trimester of pregnancy as described above.

Newborn and young animals. These animals will be exposed to two concentrations of cigarette smoke, 1. the equivalent if 1/2 of a IAI Kentucky cigarette daily for 1-2 weeks, and 2. a very low level of cigarette smoke to mimic the "passive" smoking of children.

"passive" smoking is a term to which the author does not seem to understand their

LUNG BIOCHEMISTRY

Tissue Preparation. 1 mm thick lung slices will be prepared with the Mc Ilwain tissue chopper (Brinkman Instrument Co.). It has recently been shown that this is the optimal thickness for the study of metabolism in lung slices (9).

1. GLUCOSE METABOLISM Lung slices (50-100 mg) will be incubated in 2 ml Krebs Ringer bicarbonate (KRB) buffer containing 1uCi U ^{14}C-glucose for 15-180 min; incubation will be at 37°C in a Dubnoff bath. Each incubation flask will be equipped with a center well for the collection of $^{14}CO_2$ produced during the incubation and will be aerated for 1 minute with a mixture of 95% O_2-5% CO_2 at the start of the incubation. The reaction will be stopped by the addition of 0.2 ml 30% perchloric acid; 0.2 ml phenethylamine will be injected into the fluted filter paper inside the central well and the bottles will be allowed to stand for 30-60 minutes for the complete release of $^{14}CO_2$ from the medium; the flasks will be opened, the phenethylamine soaked filter paper will be transferred to counting vials containing 10 ml Toluene Scintillator for the quantitation of the $^{14}CO_2$ formed. The tissue will be thoroughly rinsed and homogenized in chloroform:methanol-2:1 for analysis. The incubation medium will be frozen for the subsequent determination of lactate (38). The relative contribution of glycolysis and Krebs cycle vs. pentose phosphate pathway to glucose metabolism during lung development will be studied in experiments in which the tissue will be incubated separately with $1^{14}C$ glucose and $6^{14}C$ glucose and the production of $^{14}CO_2$ with the two substrates will be measured. The CO_2 production will be expressed as specific yield as suggested by Katz and Wood (39).

2. PROTEIN SYNTHESIS Lung slices (50-100 mg) will be incubated in 2 ml KRB buffer containing 2uCi of ^3H leucine. The incubation flasks will be aerated throughout the incubation (15-180 mins) with a mixture of 95% O_2-5% CO_2.

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At the end of the incubation the tissue will be rinsed and homogenized in 5 ml 10% TCA (Trichloracetic acid). The TCA precipitate will be washed x2 with 10% TCA and lipids will be extracted with a mixture of ether: acetone: chloroform- 2: 2: 1 at 50°C for 30 min. The incorporation of labeled amino acids into TCA insoluble lung protein will be quantitated by liquid scintillation spectometry and expressed as dpm /mg protein.

3. GLUCOPROTEIN SYNTHESIS Lung slices and slices of trachea will be incubated in 2 ml KRB buffer containing 2-4 4uCi ^{3}H glucosamine. Incubation will be identical to that for the incorporation of amino acids. At the end of the incubation, the tissue will be homogenized in 10% TCA + 1% Phosphotungstic acid to insure quantitative precipitation of glucoproteins. The glucoprotein precipitate will be washed X2 with 10% TCA + 1% phosphotungstic acid and processed further as described under "protein synthesis".

4. LIPID METABOLISM Uptake and esterification of ^{3}H palmitic acid will be measured also with the tissue slice preparation. ^{3}H palmitic acid will be bound to 4% albumin solution as described by Darrah and Hedley-Whyte (40). 50-100 mg lung slices will be incubated for 15-180 min in 2 ml KRB buffer (slightly modified to contain less Ca^{++}) containing 2% albumin and 0.1-0.2 uCi ^{3}H Palmitate. The following paramenters will be studied: esterification of palmitic acid, synthesis of total lipids, (neutral glycerides and phospholipids). In some experiments we will measure the amount of palmitate that is used for energy production, i.e: oxidized to CO_2, in these experiments the substrate will be $1^{14}C$ palmitic acid. The ability of the lung to synthesize phospholipids, especially lecithin, will be studied also in assay systems containing ^{3}H choline as substrate. At the end of the incubation, the tissues will be rinsed in 1% albumin solution (to remove adsorbed fatty acid) and homogenized in chloroform: methanol - 2: 1 for the analysis of lipids.

SPECIFIC ENZYMES OF SPECIAL IMPORTANCE TO INTERMEDIARY METABOLISM

Lipoprotein Lipase activity will be determined in lungs from fetal and newborn rats as described previously (32) (see Appendix) The activity will be measured in acetone-either preparations of the lungs using as substrate doubly-labeled (^{3}H-glycerol, ^{14}C-fatty aid) chylomicron triglyceride (41). Lipoprotein lipase activity will also be measured in isolated, cultured lung endothelial cells obtained in Project 3. Glucose-6-phosphate dehydrogenase activity will be measured by the method of Lohr and Waller (42).

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ENZYME ACTIVITIES AND TISSUE COMPONENTS ACTIVE IN THE METABOLISM OF CIGARETTE SMOKE INGREDIENTS

a. Aryl Hydrocarbon Hydroxylase will be measured in lung preparations by a modification (37) of the method of Nebert and Gelboin (43).

b Cytochrome P450. Methods for the quantitation of cytochrome P450 in the lung are now being developed (44). The methods are extremely elaborate and time consuming for lung tissue; we hope that in 1-2 years, at the time when we intend to embark on this specific question better methods will be available for the measurement of cytochrome P450 in the lung.

c Vitamin C will be supplemented in the diet of pregnant animals exposed to cigarette smoke; we will follow the offspring to see whether this antioxidant has a beneficial effect on the growth and lung metabolism of the newborn. (The Vit C level was found to be significantly lower in the serum of women who smoked cigarettes during pregnancy (45)).

Light and Electron Microscopy (scanning and transmission) will be performed throughout this study to follow:

1. Morphological changes during lung maturation

2. Morphological changes caused by exposure of the animals to cigarette smoke

ANALYTICAL METHODS

Analysis of tissue components The following components of the lung will be analyzed in all experimental specimens (fetal and neonatal animals; after exposure to cigarette smoke and in all samples of human tissue):

1. Protein will be measured by the method of Lowry et al (46).

2. DNA will be extracted and measured by the method of Schneider (47) or for very small tissue samples as described by Burton (48).

3. RNA will be extracted and measured according to Schneider (47) or will be calculated by subtraction of the DNA from the total nucleic acids as described by Laros et al. for human lung (49).

4. Total lipids in all tissues will be extracted and purified according to the method of Folch et al. (50).

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5. Individual lipids in the major lipid classes (neutral glycerides and phospholipids) will be separated by thin layer (40,51) and column chromotography (40,52).

6. Total lipids will be quantitated by the method of Rapport and Alonzo (53).

7. Phospholipids will be measured by the method of Bartlett (54).

Analytical methods used in metabolic studies

1. Glucose concentration will be measured with the glucose oxidase kit (Worthington).

2. Lactate concentration will be measured with the lactic dehydrogenase assay described by Hohorst (38).

3. ATP and ADP will be measured enzymatically (55).

4. Free fatty acids will be separated from esterified fatty acids by the method of Borgstrom (56).

All the methods for glucose metabolism, protein, glucoprotein, lipid and phospholipid synthesis are used at present in our laboratory. All the methods have been adapted to the study of lung metabolism during the last year and are presently used in our study on the effect of cigarette smoke on the metabolism of adult rat lung (CTR, Project 936).

In Project 2 we plan to share smoke exposed animals with Dr. Herscowitz (Department of Microbiology) who will use large numbers of mice in his project.

The methodology developed under project CTR 936, will also be applied to the biochemical studies of project 3. We will study the individual lung cell types cultured in project 3. (Type II alveolar cells, endothelial cells) for the metabolic changes induced by exposure to cigarette smoke. This will enable a comparison between the effect of cigarette smoke at organ level to that at the cellular level.

TENTATIVE SCHEDULE

1. The estimated time period for completion of part 1 (major metabolic pathways in the lung during pre and postnatal development is 12-18 months (1st and 2nd year of project).

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2. The study of specific enzymes, such as lipoprotein lipase, glucose-6-phosphate dehydrogenase (and other enzymes which will be studied as an extension of part 1) will proceed in the 2nd and 3rd year of the project. We hope that at that time cultures of the various types of lung cells will be available from project 3 and that we will be able to study the enzymes in whole lung preparations as well as in preparations of the individual cell types.

3. The effect of cigarette smoke on lung metabolism during the developmental period will be initiated as soon as we have sufficient information about the basic metabolic pathways of the control developing lung. We plan to start these smoke exposure experiments during the 2nd year of the project and to continue throughout the 5 year period.

4. Aryl hydrocarbon hydroxylase (AHH) activity, cytochrome P450 and the effect of antioxidants (Vit C) in lung of animals exposed to cigarette smoke will be studied in the 3rd -5th year of the project.

5 Human lung will be collected and studied throughout the 5 year period of the project in order to obtain a large enough sample of lungs from smokers and non-smokers (adults) or from the children of smoking and non-smoking mothers.

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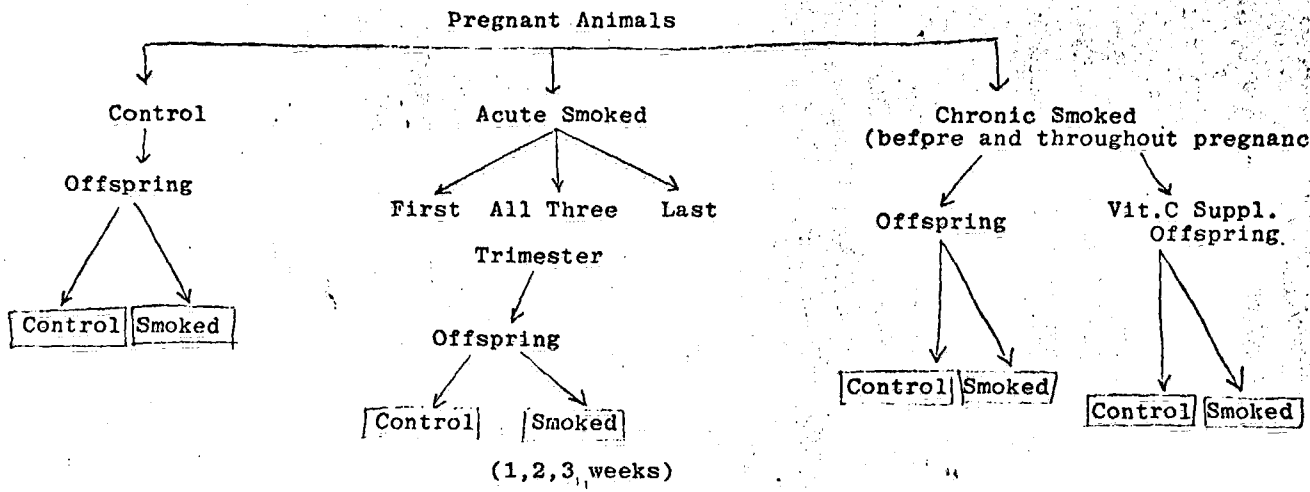
PROJECT 2 (and 3)

Smoke Exposure Design



Lung Development Design

BALB/C mice
Project 3 and/or Herscowitz

Rabbits (Dr. Bellanti)

Primates (Dr. Farrell)

Human (Hospital)

Rats

Guinea Pigs

- Metabolic Pathways → Glucose utilization, Protein synthesis; Glucoprotein synthesis, Lipid synthesis
- Enzyme Studies → Lipoprotein Lipase; Glucose-6 -Phosphate Dehydrogenase; Aryl/Hydrocarbon Hydroxylase
- Lung Composition → DNA; RNA; Protein; Lipid; Phospholipid; Cytochrome P450

P. HAMOSH M.D.

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Source: https://www.industrydocuments.ucsf.edu/docs/nndm0000

First Year Budgets
Project 2.

P. HAMOSH M.D.

13. Budget: (1st year)

A. Salaries (Personnel by names)	% time	Amount
Professional		
Dr. Margit Hamosh	100%	26,053
Post-Doctoral Fellow	100%	14,202
Technical		
Technician	100%	11,422
All Salaries Include 18.35% Fringe Benefits (16.7% 1/1/76-6/30/76 and 20% 7/1/76-12/31/76)	Sub-Total	51.677
B. Consumable Supplies (list by categories)		
Glassware and Disposables		4,000
Isotopes		2,000
Chemicals		3,000
Animals		2,000
	Sub-Total	11,000
C. Other Expenses (itemize)		
Travel		1,500
Repair and Maintanance		2,000
	Sub-Total	3,500
D. Permanent Equipment (itemize)		
Centrifuge (GLC)		1,500
E. Overhead (15% of A+B+C)		67,677
	Total	

Overhead to be negotiated at 15% or higher of A, B & C

The distribution of effort by all investigators is presented in Figure 2.

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Source: https://www.industrydocuments.ucsf.edu/docs/mnam0000